

An Engineered Cysteine in the External Mouth of a K⁺ Channel Allows Inactivation to Be Modulated by Metal Binding

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ABSTRACT Substitution of a cysteine in the extracellular mouth of the pore of the *Shaker*-Δ K⁺ channel permits allosteric inhibition of the channel by Zn²⁺ or Cd²⁺ ions at micromolar concentrations. Cd²⁺ binds weakly to the open state but drives the channel into the slow (C-type) inactivated state, which has a K_d for Cd²⁺ of ~0.2 μM. There is a 45,000-fold increase in affinity when the channel changes from open to inactivated. These results indicate that C-type inactivation involves a structural change in the external mouth of the pore. This structural change is reflected in the T449C mutant as state-dependent metal affinity, which may result either from a change in proximity of the introduced cysteine residues of the four subunits or from a change of the exposure of this residue on the surface of the protein.

INTRODUCTION

One of the remarkable properties of ion channel proteins is their ability to regulate access to their ion-selective pores through a variety of conformational changes. In many cases, these conformational changes are coupled to important stimuli such as changes in the transmembrane voltage or the binding of small signaling molecules such as neurotransmitters. The nature of these conformational changes, collectively referred to as gating, is in general not well understood. Site-directed mutagenesis studies have successfully identified many specific amino acid residues and regions of channel proteins that, when mutated, can change the kinetics of channel gating, but it can be difficult to interpret such findings in clear structural terms.

An approach combining site-directed mutagenesis with chemical modification has been used to give a more direct picture of the functional movements of proteins. The amino acid cysteine, substituted at various positions within a protein, can serve as a movable target for specific chemical modification (Falke et al., 1988; Altenbach et al., 1989; Akabas et al., 1992; Sahin-Toth and Kaback, 1993). Comparing the reactivity of cysteines at different positions gives information about their surface exposure and the transmembrane topology of the protein. In specific positions, the reactivity of a cysteine, or its ability to be disulfide-bonded or cross-linked to other cysteines, can report a change in conformation. For instance, certain disulfide bonds between introduced cysteines in the subunits of the bacterial aspartate receptor can be formed only when aspartate (or another ligand) is present to activate the receptor (Falke and Koshland,

1987). This kind of result indicates that the conformational change produces a change either in the surface exposure or in the relative proximity of introduced cysteines.

Engineered cysteines can be useful not just as targets for specific chemical reagents but also as sites of metal interaction. Cysteine is a common constituent of high affinity binding sites for metal ions, particularly for zinc and cadmium ions. Recent protein design efforts have included the production of metal binding sites with cysteine and histidine residues (Pessi et al., 1993). Naturally occurring cysteines can also be responsible for specific metal sensitivity of ion channels. A cysteine residue found in the pore-forming region of Na⁺ channels from cardiac but not skeletal muscle is responsible for the high affinity blockade of cardiac channels by cadmium ions (Schild and Moczydlowski, 1991; Backx et al., 1992; Satin et al., 1992; Heinemann et al., 1992).

The external mouth of the *Shaker* K⁺ channel is the site at which extracellularly applied tetraethylammonium (TEA) ions block current through the channel. Mutations of a single amino acid residue (at *Shaker* position 449) influence the affinity for TEA dramatically: aromatic substitutions (Phe, Tyr) at this position (normally Thr) increase the affinity, while a charged amino acid (Lys) eliminates the TEA effect completely (MacKinnon and Yellen, 1990). The *Shaker* channel is normally a homotetramer (MacKinnon, 1991); by varying the number of aromatic substitutions, Heginbotham and MacKinnon (1992) demonstrated that TEA interacts simultaneously with several subunits.

The external mouth of K⁺ channels has also been implicated in an inactivation gating process. Inactivation refers to the tendency of channels to re-close during presentation of a long activating stimulus. *Shaker* K⁺ channels inactivate by two distinct mechanisms. One of these (N-type) involves a tethered blocker or "ball and chain" that acts at the intracellular mouth of the channel (Hoshi et al., 1990). After activation, the positively charged N-terminus (inactivation ball) of the *Shaker* protein can block the internal mouth of

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the channel and occlude K⁺ current. This process is slowed when internally applied TEA binds and competes with binding of the inactivation ball (Choi et al., 1991). When the inactivation ball is removed, a slower inactivation gating (C-type) persists; this gating is sensitive to external but not internal TEA and thus involves some change at the external mouth of the pore. The rate of C-type inactivation can be altered by some mutations at position 449 or by mutations at other positions in the adjacent transmembrane region S6 (Hoshi et al., 1991; López-Barneo et al., 1993). In some cases the rate is also sensitive to external [K⁺], with higher [K⁺] tending to slow the inactivation process (Pardo et al., 1992; Labarca and MacKinnon, 1992; López-Barneo et al., 1993).

We have studied the properties of a mutant of the *Shaker* K⁺ channel with a cysteine substituted at position 449 to learn more about the external mouth of the channel and its motions during gating. Mutation of this position from threonine to cysteine has no effect per se on activation or inactivation gating rates, but the cysteine mutant is extraordinarily sensitive to Zn²⁺ or Cd²⁺ ions. We have found that this sensitivity is unlike the Cd²⁺ blockade of Na⁺ channels that contain a cysteine in the pore; it results from a strongly state-dependent interaction of Zn²⁺ or Cd²⁺ ions with the channel protein. Both onset and recovery from C-type inactivation can be modulated by Cd²⁺ through an allosteric mechanism, which reflects specific structural changes in the external mouth of the channel.

MATERIALS AND METHODS

Mutagenesis and mammalian cell expression

The T449C mutant was prepared using oligonucleotide-directed mutagenesis and the *dur⁺ ung⁻* selection scheme of Kunkel (1985) with the Mutagene kit (Bio-Rad, Richmond, CA). After mutagenesis, the mutant cDNA was subcloned into the expression plasmid GW1-CMV (British Biotech, Cowley, Oxford, UK) and completely sequenced.

For expression in mammalian cells, we used the human embryonic kidney 293 cell line (HEK293; American Type Culture Collection no. CRL-1533, Rockville, MD). Cultures were grown in DMEM-F12 (GIBCO/BRL, Gaithersburg, MD) with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) to 40–60% confluence and then split 1:3 one day before transfection. Cells for transfection were collected by treatment with 0.05% trypsin, 0.53 mM EDTA, washed once, and resuspended in HEPES-buffered saline at 2×10^6 cells/ml. Electroporation was done at 400–475 V (Electroporator, Invitrogen, San Diego, CA) in 0.4-cm cuvettes containing 200 μ l of diluted cells, 25 μ g of channel expression plasmid, 1 μ g of an SV40 T antigen expression plasmid, and 5 μ g of a β -galactosidase expression plasmid. Cells were plated onto protamine-coated coverslips and maintained in growth medium at 37°C with 5% CO₂ until use. The efficiency of transfection was monitored by staining for β -galactosidase reaction; usually 30–70% of cells were positive.

Excised patch recording

Excised outside-out patch recordings were made 20–72 h after electroporation using standard methods (Hamill et al., 1981). Pipettes (1–2 M Ω) were filled with 160 mM KCl, 1 mM EGTA, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.4. The basic bathing (external) solution was 150 mM NaCl, 10 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4. CdCl₂ and ZnCl₂ (both 99.999% pure; Aldrich) were added to this solution at the indicated concentrations. The control solution used in all experiments with Cd²⁺ was

the basic solution plus an additional 1 mM CaCl₂ and 1 mM EGTA, with the pH adjusted after addition to 7.4. Since EGTA has a higher affinity for Cd²⁺ than for Ca²⁺, this wash solution chelates any leftover Cd²⁺.

We used the rapid perfusion method of solenoid-switched converging streams described by Brett et al. (1986). Control experiments using external TEA to block K⁺ current showed that perfusion was complete in 1–3 ms. Electrical recording was accomplished using an Axopatch 200A patch clamp (Axon Instruments, Foster City, CA). The holding potential in all experiments was –80 mV; all test depolarizations were to 0 mV. Saline-filled salt bridges connected the bath with a Ag-AgCl grounding wire.

Data collection and analysis

Control of membrane voltage, perfusion, and data acquisition were performed on a 486-based PC equipped with an AT-MIO-16F-5 analog interface (National Instruments, Austin, TX) using our own Windows-based software (VectorView; G. Yellen, unpublished). Analysis of exponential decays was done either by eye or by a Levenberg-Marquardt minimization (Origin software, MicroCal, Northampton, MA).

RESULTS

Cysteine substitution in the outer mouth confers sensitivity to Zn and Cd

Fig. 1 shows the response of wild-type *Shaker*- Δ channels and the T449C mutant to 300 μ M Zn²⁺ or Cd²⁺. The channels are studied in excised outside-out patches from mammalian cells that were transiently transfected with channel

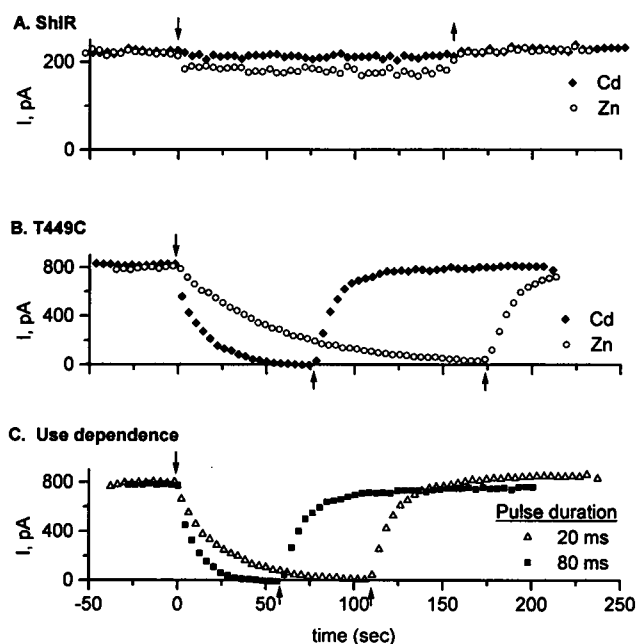


FIGURE 1 Mutation of *Shaker* position 449 to cysteine confers sensitivity to Zn²⁺ and Cd²⁺. (A) Effect of 300 μ M Zn²⁺ (open symbols) or Cd²⁺ (solid symbols) on the wild-type (Sh Δ) channel. Symbols represent the average current during the second half of a voltage clamp depolarization to 0 mV, lasting 40 ms. Pulses were applied every 4 s; metals were added at the time indicated by the down arrows, and removed at the up arrows. (B) Effect of 300 μ M Zn²⁺ or Cd²⁺ on the T449C mutant channel, performed as in A. (C) Use-dependence of the Cd²⁺ effect on T449C. Experiment as in B, except that the pulse duration was changed to 20 ms (open symbols) or 80 ms (solid symbols).

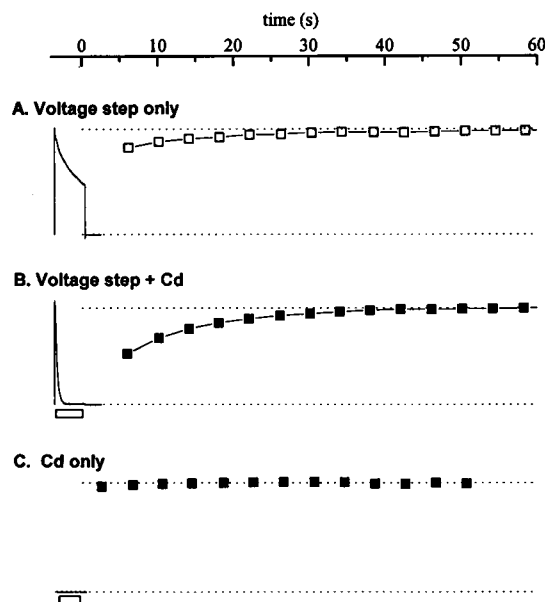


FIGURE 2 State-dependence of the Cd^{2+} effect on T449C channels. (A) Response to a long depolarization showing slow (C-type) inactivation, followed by a series of brief pulses to monitor recovery. (B) Cd^{2+} effect on open channels. Experiment as in A, except that $300 \mu\text{M}$ Cd^{2+} was applied for 4 s beginning 100 ms after start of depolarization. (C) Cd^{2+} effect on closed channels. $300 \mu\text{M}$ Cd^{2+} was applied for 3.5 s while maintaining V_m at -80 mV. Any long-lasting effect should be seen as a reduction in current during the recovery pulses.

expression plasmids; expression levels are so high that macroscopic currents (arising from hundreds to thousands of channels) are observed in the patches. All channels are from an N-terminal deletion mutant ($\Delta 6-46$; Sh Δ ; Hoshi et al., 1990) that lacks N-type inactivation. The metals are applied during repeated brief depolarizations to 0 mV.

The small effect of these ions on the wild-type Sh Δ channel is a consequence of a slowing of wild-type K^+ channel activation gating (Gilly and Armstrong, 1982; Boland et al., 1994), an effect seen particularly with Zn^{2+} . The effects on the T449C mutant channel are much more dramatic. Currents through the mutant channel are completely inhibited by these concentrations of Zn^{2+} or Cd^{2+} . The inhibition requires about a minute for completion, and it is completely reversed after 30–40 s of washing with a control solution containing Ca-EDTA or Ca-EGTA (which chelates any remaining Zn^{2+} or Cd^{2+}).

Because Cd^{2+} is less potent than Zn^{2+} in producing the slowing of activation, we have mostly used Cd^{2+} to investigate the mechanism of inhibition of the T449C mutant. All of the effects described here for Cd^{2+} are seen also with Zn^{2+} at comparable concentrations.

The slow onset of the inhibition made us suspect that the metal effect on T449C was use-dependent. Use-dependence is commonly seen with channel inhibitors that preferentially bind to a particular gating state of the channel; a simple example is open-channel blockade, in which a blocking agent can only reach its blocking site when the channel is open.

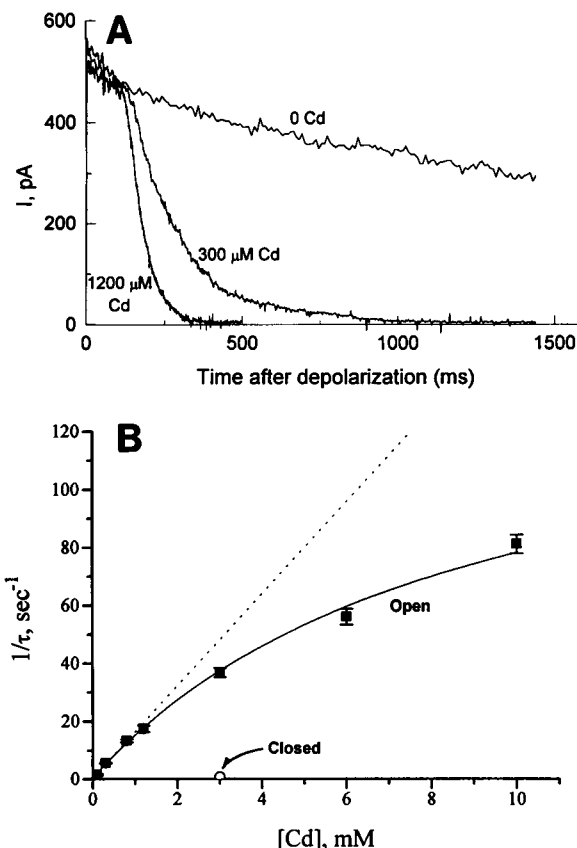


FIGURE 3 Inhibition of open T449C channels by Cd^{2+} . (A) The indicated concentration of Cd^{2+} was applied during a long depolarization to 0 mV, beginning 130 ms after depolarization. (B) The rate of open channel inhibition is plotted as a function of $[\text{Cd}^{2+}]$. The solid symbols are derived from experiments as in A. Each symbol represents the mean \pm SEM of rates measured on at least three patches. The solid line is a minimized fit to the equation $r = (r_0 \cdot K + r_{\text{Cd}} [\text{Cd}]) / (K + [\text{Cd}])$, where r_0 is the rate without Cd^{2+} , r_{Cd} is the maximum rate with Cd^{2+} , and K is the apparent dissociation constant for Cd^{2+} . The value for r_0 was fixed at 0.6 sec^{-1} , the rate of C-type inactivation without Cd^{2+} . The fitted values for r_{Cd} and K were $150 \pm 10 \text{ sec}^{-1}$ and $9 \pm 1 \text{ mM}$, respectively. The dotted line is the best linear fit to the rates measured between 0 and 1 mM Cd^{2+} ; it has a slope of $1.6 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. The open symbol is the inferred rate for closed channel inhibition by 3 mM Cd^{2+} , determined from the data in Fig. 4.

Use-dependence is manifest as a slow rate of inhibition that can be altered by the frequency or duration of channel activation. Fig. 1 C shows that the Cd^{2+} effect on T449C is indeed use-dependent: longer pulses speed the onset of Cd^{2+} inhibition.

Inhibition by Cd^{2+} is enhanced in the open state

We used a computer-controlled rapid perfusion system to apply Cd^{2+} at specific times to determine which states of the channel are inhibited by Cd^{2+} . In the absence of Cd^{2+} , a 4-s depolarization to 0 mV evokes an outward current (Fig. 2 A) that then declines slowly with time due to C-type inactivation ($\tau \approx 1.6$ s). When $300 \mu\text{M}$ Cd^{2+} is applied during the long depolarizing pulse (Fig. 2 B), the current declines very rapidly ($\tau \approx 160$ ms) to 0. Clearly the effect of Cd^{2+} is much

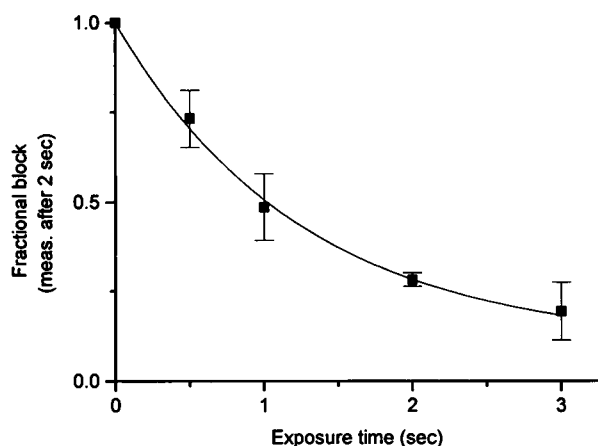


FIGURE 4 Inhibition of closed T449C channels by 3 mM Cd²⁺. Experiments were performed as in Fig. 2 C. A patch held at -80 mV was exposed for the indicated length of time to 3 mM Cd²⁺; 2 s after the removal of Cd²⁺, a brief depolarizing pulse was applied to assess the long-lasting effect of the Cd²⁺ application. The current in this test pulse, normalized to a test pulse without Cd²⁺ exposure, is plotted as a function of the duration of exposure. The solid line is a best fit exponential ($y = (1 - y_0)\exp(-t/\tau) + y_0$) with $\tau = 1.2 \pm 0.5$ s and $y_0 = 0.1 \pm 0.14$. For comparison with open channel inhibition, the rate $1/\tau$ is plotted in Fig. 3 B as the open circle.

faster when applied during a long depolarizing pulse, as compared with the repeated brief pulses in Fig. 1. Recovery from both inactivation and Cd²⁺ inhibition, as monitored by a series of brief depolarizations during the recovery period, is slow and occurs with a time constant of about 10 s.

Fig. 3 A shows rapid inhibition of open channels by two concentrations of Cd²⁺ on an expanded time scale. The inhibition was generally well-described by a single exponential function, whose rate increased with increasing concentrations of Cd²⁺. The rate of decay is plotted in Fig. 3 B as a function of [Cd²⁺]. Two important features of the open state inhibition are apparent from this plot. First, the rate of inhibition tends to saturate at high [Cd²⁺]; the data are fit by a rectangular hyperbola with $K_{1/2} = 9 \pm 1$ mM and a maximum rate of 150 ± 10 sec⁻¹. Second, the rate of inhibition at limiting low concentration is extremely small (1.6×10^4 M⁻¹ sec⁻¹) compared with diffusion limitation ($\approx 10^{10}$ sec⁻¹). Both properties are inconsistent with a simple bimolecular reaction leading to channel blockade ($O + Cd \rightarrow O \cdot Cd$), which should have a high on-rate that continues to increase linearly at high [Cd²⁺].

The effect of Cd²⁺ on closed channels was much smaller and could only be observed indirectly. Cd²⁺ was applied while the channels remained closed at -80 mV; any long-lasting effect of Cd²⁺ should appear as a reduction in the subsequent brief pulses to 0 mV. When 300 μ M Cd was applied for 3.5 s (Fig. 2 C), no long-lasting inhibition was observed; however, a higher concentration can produce long-lasting inhibition even when the voltage is held at -80 mV. We applied 3 mM Cd²⁺ for varying times and monitored the amplitude of a test pulse applied 2 s after the removal of Cd²⁺ (Fig. 4). This gives a rate of about 0.8 sec⁻¹ for inhibition of

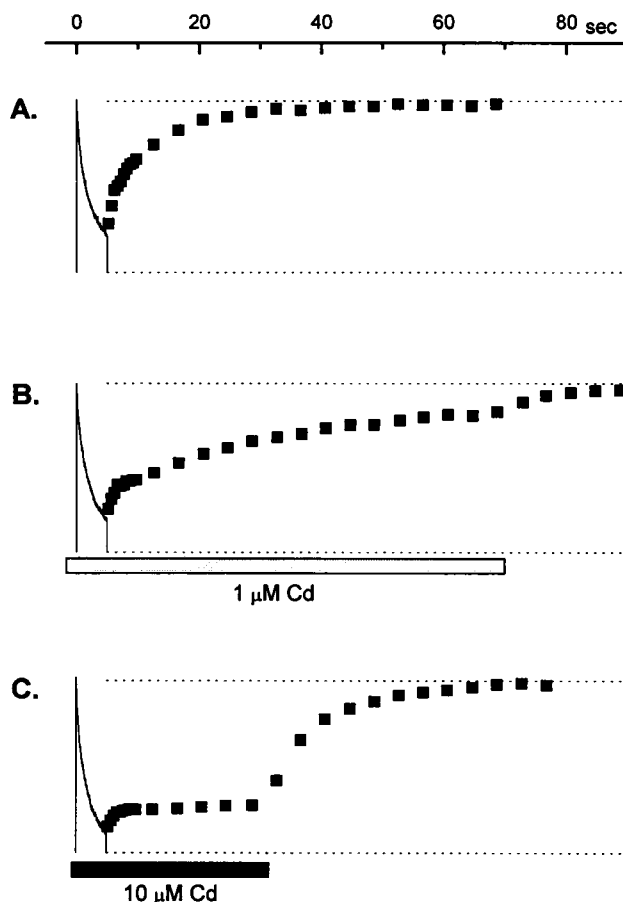


FIGURE 5 Recovery from C-type inactivation is slowed by very low [Cd²⁺]. Following a 5-s depolarization (current trace) that inactivates many of the channels, brief depolarizations are applied to monitor recovery from inactivation (symbols). In B and C, the indicated concentration of Cd²⁺ is applied during the period indicated by the bar.

closed channels at this high [Cd²⁺], which is much slower than inhibition of open channels (see open circle in Fig. 3 B).

Recovery from C-type inactivation is slowed by very low [Cd]

Because of earlier experiments showing that external TEA binding affects the rate of C-type inactivation (Choi et al., 1991), we suspected that the T449C mutant channel might show an altered interaction with Cd²⁺ when in this inactivated state. We examined this interaction by measuring the rate of recovery from inactivation in the presence and absence of Cd²⁺. After a long depolarizing pulse that allowed about 70% of the channels to inactivate, recovery was monitored by a series of brief depolarizing pulses (Fig. 5 A). Recovery occurs in two phases. About one-fourth of the inactivated channels recover in less than a second; the remaining channels recover with a time constant of about 10 s. In the presence of a very low [Cd²⁺] of 1 μ M, the slow phase of recovery is markedly slower (Fig. 5 B); with 10 μ M Cd²⁺,

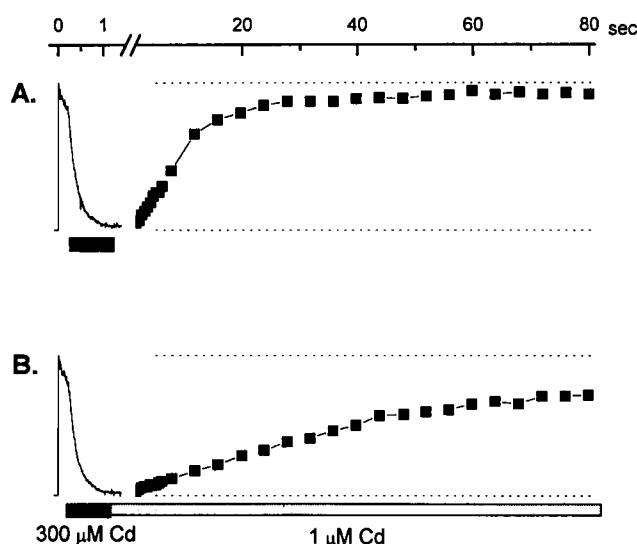


FIGURE 6 Recovery from Cd^{2+} inhibition of the open channel is also slowed by very low $[\text{Cd}^{2+}]$. Channels were opened by a 1-s depolarization to 0 mV and inhibited by a pulse of $300 \mu\text{M}$ Cd^{2+} . Recovery was monitored by brief depolarizing pulses, as in Fig. 5. In B, $1 \mu\text{M}$ Cd^{2+} was present during the recovery period. In this experiment, Cd^{2+} was removed after 200 s and the current recovered to 100% of its original value.

the channel is practically frozen in the inactivated state but recovers promptly (at the original rate) when Cd^{2+} is removed (Fig. 5 C).

The presence of two phases to the recovery from long-pulse-induced inactivation indicates that the C-type inactivated state actually consists of at least two inactivated states: one that recovers slowly and is affected by Cd^{2+} , and another that recovers rapidly and is not affected by Cd^{2+} . In our subsequent analysis we have restricted our attention to the slowly recovering state.

Cd^{2+} inhibition of the open state forces the channel to the C-type inactivated state

The Cd^{2+} inhibition of the open state recovers with an exponential time course at approximately the same rate as the slow phase of recovery from C-type inactivation. We therefore tested to see whether low $[\text{Cd}^{2+}]$ could affect this recovery process in a similar fashion. A pulse of $300 \mu\text{M}$ Cd^{2+} was applied during depolarization, and recovery in the presence or absence of $1 \mu\text{M}$ Cd^{2+} was monitored by brief pulses (Fig. 6). Just as for recovery from long-pulse-induced inactivation, $1 \mu\text{M}$ Cd^{2+} substantially slows the recovery from Cd^{2+} inhibition.

Both the absolute rate of recovery and the effect of Cd^{2+} on this rate are the same for long-pulse-induced inactivation (the slowly recovering fraction) and for Cd^{2+} inhibition. We therefore hypothesize that Cd^{2+} , when applied to the open T449C channel, induces a transition to the C-type inactivated state. Long-pulse-induced inactivation places the channel in a mixture of states; one recovers rapidly and the other more

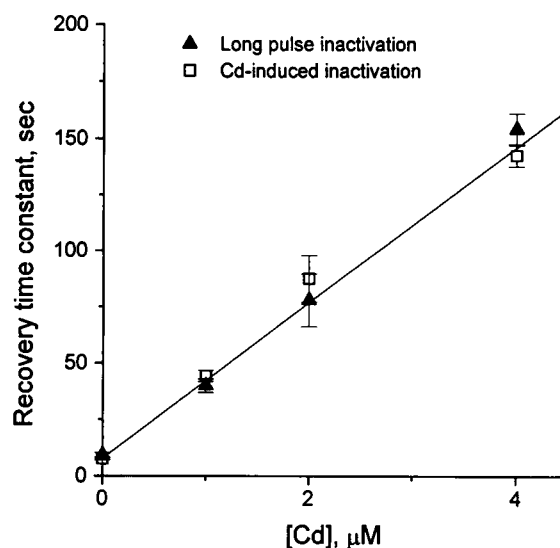


FIGURE 7 Recovery from long-pulse- or Cd^{2+} -induced inactivation as a function of $[\text{Cd}^{2+}]$. The recovery time constant for long-pulse-induced inactivation (Fig. 5) or Cd^{2+} -induced inactivation (Fig. 6) is plotted as a function of $[\text{Cd}^{2+}]$. For long-pulse inactivation, the recovery time constant is derived only from the slower recovering fraction of channels. Each symbol represents the mean \pm SEM for measurements on at least three patches. The best fit straight line has an intercept $\tau_0 = 7.6 \pm 0.6$ s, and the inverse of the slope is $K_{\text{Cd}} = 220 \pm 20$ nM (see scheme 1 in the text).

slowly. " Cd^{2+} -induced inactivation" places the channel entirely in the more slowly recovering form.

An alternative model, in which Cd^{2+} waits for the channel to enter the inactivated state, cannot account for the open state inhibition. The rate of entry into the inactivated state can be no faster than the inactivation relaxation rate in the absence of Cd^{2+} , which is 0.6 sec^{-1} . Even if Cd^{2+} reduces the recovery rate to 0, the rapid inhibition seen at high Cd^{2+} would not be seen unless Cd^{2+} also interacts with the open state.

The effect of Cd^{2+} on recovery from inactivation exhibits very high affinity. Fig. 7 shows the recovery time constant as a function of $[\text{Cd}^{2+}]$ for both long-pulse- and Cd^{2+} -induced inactivation. Recovery time gets progressively longer at higher $[\text{Cd}^{2+}]$, consistent with a scheme in which Cd^{2+} binding to the inactivated state prevents recovery:



In this scheme, the recovery time constant in the absence of Cd^{2+} is $\tau_0 = \lambda^{-1}$. As $[\text{Cd}^{2+}]$ is raised, recovery gets slower with $\tau = \tau_0(1 + [\text{Cd}^{2+}]/K)$, where K is the dissociation constant for Cd^{2+} binding to the inactivated state. Applying this scheme to the straight line fit of τ vs. $[\text{Cd}^{2+}]$ in Fig. 7 gives $K = 220 \pm 20$ nM, an affinity that is 45,000-fold tighter than the $K_{1/2}$ for the effect of Cd^{2+} on the open state.

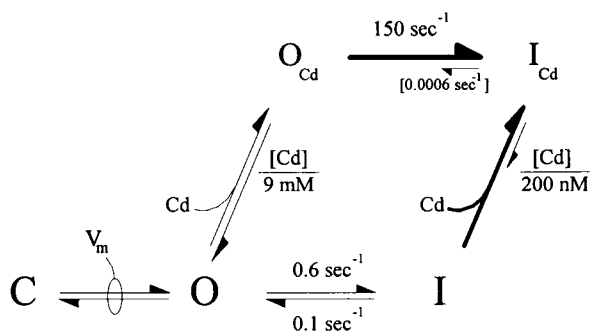


FIGURE 8 A kinetic scheme to account for the effects of Cd²⁺ on C-type inactivation of the T449C channel. A detailed description of the scheme appears in the text. The rate and equilibrium constants shown are derived from data in Figs. 3 B and 7. Equilibrium constants are indicated for Cd²⁺ binding to the O and I states as [Cd]/K_{Cd}. The C state shown here represents multiple closed states that are connected to the open state by both voltage-dependent and voltage-independent transitions. The I_{Cd} → O_{Cd} rate, in brackets, is inferred from the thermodynamic constraints on this cyclic scheme. The scheme does not include a state to account for the rapidly recovering fraction of channels noted in Fig. 5.

DISCUSSION

Conformation-sensitive binding of Cd explains the allosteric effect on inactivation

We have found that with a cysteine at position 449 of the ShΔ channel, Cd²⁺ has two effects that are not seen with the wild-type channel. The most potent effect of Cd²⁺ is to inhibit recovery from the slowly recovering C-type inactivated state by binding with very high affinity (220 nM) to this state. The other effect of Cd²⁺ is to speed the rate of entry into this inactivated state; this effect occurs with substantially lower affinity (9 mM). Both effects are well described by a kinetic scheme (Fig. 8) with a single, very stable inactivated state with Cd²⁺ bound (I_{Cd}). This state can be reached from the open state by two paths: inactivation followed by Cd²⁺ binding, or binding followed by inactivation. When inactivation precedes binding, the binding occurs with high affinity and prevents recovery according to scheme 1 above. When Cd²⁺ is applied to the open channel, binding occurs with low affinity, but the subsequent conformational transition to the I_{Cd} state is highly favored and occurs quickly. Effects on the closed channel are much slower, indicating that prior opening of the channel is necessary either for the low affinity binding or for the subsequent transition to the I_{Cd} state.

This allosteric effect of Cd²⁺ is a consequence of the very large change in Cd²⁺ affinity that occurs upon inactivation gating. The 45,000-fold change in affinity occurs without entrapment of the Cd²⁺ ion: binding and unbinding appear to be rapid, even for the high affinity state. When Cd²⁺ is removed during recovery from inactivation, recovery promptly resumes its normal Cd²⁺-free rate (Fig. 5, B and C), indicating that there is no long-lasting effect of Cd²⁺.

The shift in affinity for Cd that occurs upon inactivation gating (10^{4.7}) is fairly large by comparison with allosteric

shifts in ligand binding to other proteins. For example, diphosphoglycerate binding to hemoglobin prefers the deoxy form by about 20- to 400-fold, depending on the conditions of measurement (Kilmartin and Rossi-Bernardi, 1973); ouabain has a range of affinities for various conformations of the Na, K-ATPase, varying over a range of about 10³ (Wallick and Schwartz, 1988). Similar allosteric changes in binding have also been seen with ion channels: acetylcholine receptors change their affinity for acetylcholine by a factor of about 10⁴ upon desensitization (Changeux et al., 1984), and a chloride channel from *Torpedo* electric organ changes its affinity for H⁺ by a factor of 10³ upon opening (Hanke and Miller, 1983).

Comparison with other metal-thiolate binding sites

To understand the nature of the metal binding site in the T449C mutant channel, it is informative to compare its various affinities to that of other metal-thiolate binding sites. Though Cd²⁺ and Zn²⁺ bind particularly avidly to thiols, the presence of other coordinating ligands (nitrogen, oxygen, or even other sulfur atoms) can increase binding affinity substantially. For instance, β-mercaptoethanol, with —SH and —OH groups, has an affinity for Cd²⁺ of 80 μM; 2-aminoethanethiol, with —SH and —NH₂ groups, has a Cd²⁺ affinity of 0.1 μM (Martell and Smith, 1974; computed at pH 7.4). Many proteins that regulate DNA transcription have a “zinc finger” motif, which employs cysteines and histidines in a high-affinity Zn²⁺ site; model zinc finger peptides that contain four coordinating cysteine residues have an affinity for Cd²⁺ of 4·10⁻¹⁴ M (Krzek et al., 1993).

The cardiac Na channel, which probably has only a single cysteine in the pore, has an affinity for Cd²⁺ of 20 μM (Schild and Moczydlowski, 1991); it is not known what other ligands may help to coordinate the Cd²⁺.

In addition to the identity of the coordinating atoms, there are many other factors that can influence the affinity of Cd²⁺ binding to a protein. Among these are the electrostatic potential at the binding site, the pK_a of the coordinating groups, steric accessibility, proximity of the coordinating groups to one another, and the degree of strain that may be introduced into the protein to accommodate the metal ion. Moreover, because of cadmium's propensity to complex with many types of solute ligands as well as with water, the concentration of free Cd²⁺ is quite uncertain. We have referred all of our rates and affinities to the concentration of added Cd²⁺, so that the association rate constants that we report are lower limits and the affinity constants are upper limits for the true values.

Conformational effects on binding may occur through changes in proximity or accessibility

We propose two alternative structural models to explain the large change in Cd²⁺ affinity that occurs upon inactivation. The first involves a change in the relative proximity of

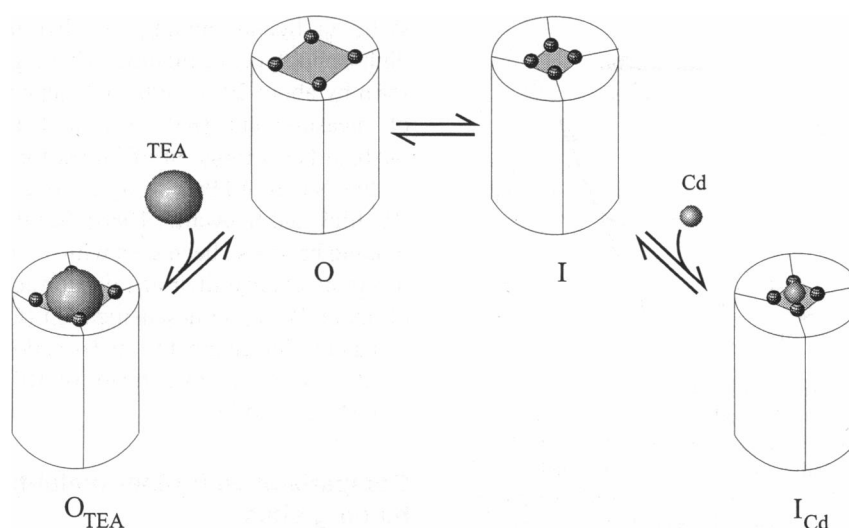


FIGURE 9 Inactivation may change the proximity of the four 449 cysteines. This model suggests that the cysteines at position 449 in the four subunits of the T449C channel may come closer together in the C-type inactivated state. The cysteine sulfurs are the four small balls attached to the channel subunits. As indicated, TEA binding prevents inactivation, but Cd^{2+} binds better to the inactivated state.

the four cysteines at the 449 positions of the four subunits (Fig. 9). The essential feature of this model is that these four cysteines are relatively far apart in the open state and permit low affinity binding of the Cd^{2+} to only one cysteine at a time; upon inactivation the cysteines come closer together, allowing high affinity binding of the Cd^{2+} to several cysteines at once. This model is suggested by the earlier observation that the C-type inactivation of the ShA channel is slowed in the presence of external TEA (Choi et al., 1991), as though TEA binding prevents the closing of a gate at the external mouth of the channel. This model also draws support from the picture of high affinity TEA binding proposed by Heginbotham and MacKinnon (1992). They found that varying the number of channel subunits with a mutation at the 449 position affects the energy of TEA binding in an additive fashion, suggesting that the TEA molecule (0.8 nm diameter) can interact simultaneously with all four subunits at or near the 449 site. In our model, TEA binds to the open (non-inactivated) state and prevents the outer mouth from constricting and entering the inactivated state; after constriction, the smaller Cd^{2+} ion (0.2 nm diameter) binds the T449C channel with improved affinity.

Our second model is that the change in affinity occurs because of a change in the exposure of the T449C cysteine at the surface of the protein (Fig. 10). In the open channel, the cysteine side chains are relatively buried and bind Cd^{2+} with only low affinity; upon inactivation, the cysteine sulfurs become exposed and capable of binding with higher affinity. An attractive feature of this model is the ready explanation that it provides for many of the mutational effects on C-type inactivation. Mutations at the 449 site can make C-type inactivation occur faster or slower than normal; hydrophobic residues (tyrosine, valine, uncharged histidine) tend to stabilize the open state, strongly polar residues (glutamate, lysine, histidine⁺) destabilize the open state and favor inactivation, and small neutral residues (threonine and cysteine)

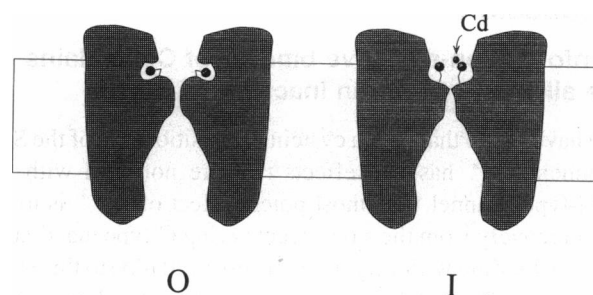


FIGURE 10 Inactivation may change the surface exposure of the 449 cysteines. An alternative to the model in Fig. 9, this model suggests that the conformation-dependent change in Cd^{2+} affinity is a consequence of the exposure, upon inactivation, of normally buried cysteine side chains.

are intermediate (López-Barneo et al., 1993; this study for cysteine). Stability of the open state is roughly consistent with the tendency of the residue at position 449 to remain buried, as our second model suggests.

The two models we propose are not mutually exclusive; the cysteine side chains might become unburied and closer together at the same time. We are presently working on experimental tests to distinguish which of these effects may contribute to the large change in Cd^{2+} affinity upon inactivation; we think that the results of these tests will also give insight into the nature of the structural change in the outer mouth of the channel that accompanies C-type inactivation.

Possible physiological implications

We have seen an interesting and potent modulation by zinc or cadmium ions of the inactivation of a mutant K^+ channel. Does such modulation ever occur in nature? Zinc ions are released from CNS synapses in a Ca^{2+} -dependent fashion, and the concentrations achieved are adequate both for the use-dependent inhibition of our mutant and for a dramatic

slowing of the recovery from C-type inactivation (Assaf and Chung, 1984; Smart, 1990). Might naturally occurring K⁺ channels respond to this Zn²⁺ in the way that we have seen for the T449C mutant?

Two cloned K⁺ channels have a cysteine at a position homologous to *Shaker* position 449. One is the *Shab* channel from *Drosophila*, a slow voltage-activated K⁺ channel (Wei et al., 1990; Pak et al., 1991). This channel, when expressed in *Xenopus* oocytes, can be inhibited completely by 300 μ M Zn²⁺ or Cd²⁺ (data not shown); we do not know whether the *Shab* channel ever encounters this concentration of Zn²⁺ in its environment in the fly. A second cloned channel with a cysteine at this position is the more distantly related *IRK1* inward rectifier K⁺ channel (Kubo et al., 1993). The sensitivity of this channel to Zn²⁺ or Cd²⁺ has not been reported, although a similar channel in crayfish muscle is found to have a high sensitivity to Cd²⁺ (Araque and Buño, 1991). Again, it is not known whether the *IRK1* channel experiences changes in [Zn²⁺] when in the native macrophage membrane. Other cloned K⁺ channels have a histidine at the position homologous to *Shaker* position 449. Since histidine participates together with cysteine in coordinating Zn²⁺ binding to zinc finger proteins, it is possible that these channels will also show use-dependent inhibition by Zn²⁺. It will be interesting to see, as more K⁺ channel cDNAs are cloned, if any of the K⁺ channels found in areas of mammalian brain with substantial reserves of Zn²⁺ are modulated through the allosteric mechanism seen for the T449C mutant.

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